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Abiotic Protein Fragmentation by Manganese Oxide: Implications for a Mechanism to Supply Soil Biota with Oligopeptides

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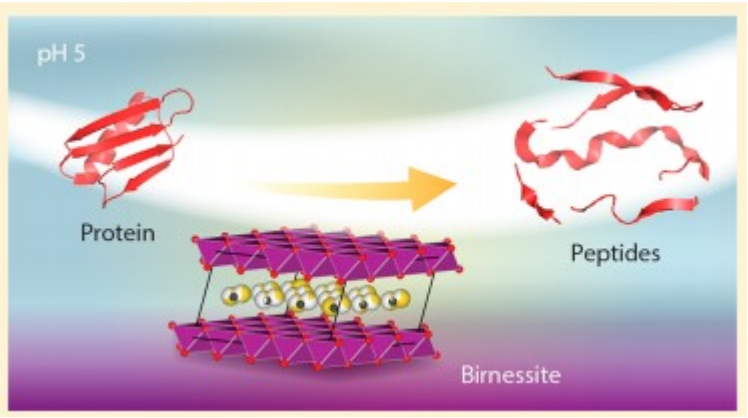
Abstract

The ability of plants and microorganisms to take up organic nitrogen in the form of free amino acids and oligopeptides has received increasing attention over the last two decades, yet the mechanisms for the formation of such compounds in soil environments remain poorly understood. We used Nuclear Magnetic Resonance (NMR) and Electron Paramagnetic Resonance (EPR) spectroscopies to distinguish the reaction of a model protein with a pedogenic oxide (Birnessite, MnO_2) from its response to a phyllosilicate (Kaolinite). Our data demonstrate that birnessite fragments the model protein while kaolinite does not, resulting in soluble peptides that would be available to soil biota and confirming the existence of an abiotic pathway for the formation of organic nitrogen compounds for direct uptake by plants and microorganisms. The absence of reduced Mn(II) in the solution suggests that birnessite acts as a catalyst rather than an oxidant in this reaction. NMR and EPR spectroscopies are shown to be valuable tools to observe these reactions and capture the extent of protein transformation together with the extent of mineral response.



1 Introduction

Patterns of abundance and diversity of proteins are considered to be critical indicators of soil ecosystem function.(1) Recent attempts at understanding

the composition and functional status of soil proteins have been directed toward two major objectives: First, information on *enzyme production and turnover*, *enzyme kinetics*, *and interactions with soil and sediment matrices* has been sought with the intent to obtain parameters for biogeochemical C cycling models.(2-4) Second, integrated *proteomic–genomic approaches* are considered necessary to link specific physiological processes to species diversity in terrestrial ecosystems.(5-7) Both objectives require not only detection and quantification of soil proteins, but also assessment of protein functional status.

Achieving these objectives is seriously confounded by the propensity of proteins to associate strongly with soil mineral and organic components through multiple processes. These include redistribution of surface associated ions, dispersion forces between the protein and the sorbent material, changes in the hydration of the sorbent surface and the protein, electrostatic interactions, the hydrophobic effect and structural rearrangements in the protein molecule.(8, 9) Elucidation of these interactions leads to the identification of controls on protein adsorption, such as pH, ionic strength and species of counterions present. Studies have shown that proteins can retain activity after adsorption even when structural rearrangement may have occurred, potentially allowing an adsorbed exoenzyme to remain functional while benefiting from the physical protection provided by a mineral sorbent surface.(8)

While substantial research efforts have been invested in understanding protein–mineral adsorption processes and the concomitant alterations to protein conformation, much less is known about the ability of mineral surfaces to induce chemical alterations to proteins that go beyond conformational change. Proteins are generally susceptible to oxidative damage, with intracellular oxidation facilitated by reactive oxygen species and other radicals.(10) In aquatic systems, indirect photooxidation by singlet oxygen can also contribute to oxidative damage of proteins.(11) Exoenzyme oxidation in soils can occur through interactions with reactive oxygen species derived from Fenton type reactions of transition metals or peroxidase enzymes with H_2O_2 produced by soil microorganisms,(12-15) but may also result from contact with soil minerals. However, while the pedogenic oxides of manganese are known for their oxidative power toward organic compounds,(16-18) reports on the effects of manganese oxide on proteins are limited to a report of apparent oxidative degradation of a prion protein in the presence of birnessite.(19)

The potential role of manganese oxides in protein degradation is particularly relevant considering that Manganese is the 12^{th} most abundant element in the earth’s crust,(20) and manganese oxides are widely disseminated in soils of different ages and from different parent materials. They frequently occur as black nodules one or two mm in diameter or as coatings on peds, sometimes as small patches or dendrites in seasonally wet, imperfectly drained soils.(21) Manganese oxides are more abundant in soils formed from

mafic rocks(22) than siliceous ones. Limestone derived soils often contain appreciable concentrations of Mn-oxide nodules.(21) Reduced Mn (Mn^{2+}) is very soluble and is mobile in soils. Consequently, black Mn oxides frequently form on the exterior surfaces of weathered rocks, particularly in moderate to dry climates.(21) Moreover, recent reports have shown that manganese loading from industrial inputs, such as steel manufacturing, coal fired power plants, and gasoline combustion have contributed to soil manganese enrichment.(23-25)

The conceptual idea behind this work was to assess the scope of alterations a protein might experience by exposing it to two archetypes of mineral surface reactivity. The first mineral we chose was kaolinite because of its low layer charge and role as a classic adsorbent of proteins. Kaolinite serves as a support in reversible exchange but is not expected to act as a reactant in a chemical reaction with proteins.(26-28) The second mineral chosen was the pedogenic oxide birnessite, which is known for its ability to actively oxidize a plethora of organic compounds.

The combination of two spectroscopic techniques with the ability to observe key reactant features allowed us to monitor the persistence of protein structure as reaction conditions are varied. Nuclear Magnetic Resonance (NMR) Spectroscopy provides a sensitive and nondestructive method to examine structural modifications to the protein, while Electron Paramagnetic Resonance (EPR) Spectroscopy quantifies the extent to which Mn(II) was released into solution during the reaction of protein with birnessite.(18)

2 Materials and Methods

2.1 Preparation of Protein–Mineral Samples

The model protein chosen for this study is the B1 domain from streptococcus Protein G. This protein was chosen because it is a small, 56 residue, stable protein that has been extensively characterized by NMR spectroscopy and been used as a model protein for other studies.(29-33) It contains well-defined secondary structure, including β-sheet and α-helix. The theoretical isoelectric point (pI) of Gb1 was calculated using ProtParam.(34) Isotopically labeled as well as natural abundance B1 domain from streptococcus Protein G (Gb1) was prepared using a method similar to that previously reported(29) and as described in detail in the Supporting Information (SI). Preparation and characterization of the minerals is available in the SI. Purified Gb1 was diluted in 5 mM sodium acetate (pH 5.0), 5 mM sodium phosphate (pH 7.0) buffer or water to a concentration of 0.4 mg/mL. Buffers were used in some experiments but were found to have no significant impact on the results, as shown in the SI. Stable isotope labeled Gb1 was used for NMR samples and natural abundance Gb1 was used for other techniques. The Gb1 solution was mixed with solid minerals in a 0.4:20 mg ratio of Gb1/mineral. The pH of each sample was checked and adjusted to target pH values of 5 and 7. The mixtures were reacted at room temperature with continuous shaking and aliquots removed at the specified times. For experiments conducted under

anaerobic conditions, Gb1 protein and birnessite were prepared separately in 5 mM sodium acetate and the pH adjusted as needed. The two solutions were mixed in an anaerobic chamber and aliquots removed at the specified time points. Samples were centrifuged for 5 min at 21 000×*g* at 4 °C and supernatants were retained for subsequent analysis. Time course experiments were conducted in 4 mL total volume with 20 mg/mL birnessite and 0.4 mg/mL final concentration of labeled Gb1 protein. The experiments were carried out in water adjusted to the desired pH. Aliquots of 400 µL were removed from the reaction, centrifuged at 21 000×*g* at 4 °C and supernatants retained for subsequent analysis.

2.2 NMR Spectroscopy

Solution state NMR experiments were performed on 800 or 600 MHz Agilent VNMRS spectrometers equipped with a cryogenic HCN triple resonance probe. D_2O and 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) were added to each solution state NMR sample to a final concentration of 10% v/v and 0.5 mM respectively. Data were collected at 25 °C. ^{15}N-HSQC spectra were collected with 1024 complex points in the direct (^{1}H) dimension and 128 complex points in the indirect (^{15}N) dimension. Triple resonance NMR experiments (HNCACB, HNCA, and HNCO) were employed to assign amino acid resonances. These experiments were conducted using nonuniform sampling techniques and reconstructed using SCRUB software.(30, 35, 36) NMR spectra were processed using NMRPipe(37) and visualized using NMRViewJ.(38) To confirm fragmentation observed in the NMR experiments, aliquots of the NMR samples were analyzed with Tris-Tricine SDS-PAGE.(39)

2.3 EPR Spectroscopy

Electron paramagnetic resonance (EPR) spectra were acquired with a Bruker Elexsys 580 spectrometer fitted with an SHQE resonator. Room temperature aqueous samples were contained in 1.0 mm OD × 0.8 mm ID quartz tubes (Vitrocom), which, for ease of handling, were placed in conventional 4 mm OD × 3 mm ID quartz EPR tubes (Wilmad). The microwave frequency was ~9.86 GHz (X band) with a power of 20 mW. The field was swept at 1000 G (G) in 84 s and modulated at a frequency of 100 kHz with 5 G amplitude. A time constant of 82 ms was employed, and typically 20 scans were averaged. Because the traditional method of quantitation—double integration of the EPR spectrum—introduced large errors from baseline subtraction at the limit of detection, an alternate method was used. A Labview program was used to abstract the peak to peak amplitude of the penultimate hyperfine line, which was converted to concentration via a conversion factor. This conversion factor was obtained from a calibration curve for Mn^{2+} using $MnCl_2$ solutions created in volumetric glassware.

3 Results and Discussion

The 2D ^{15}N-HSQC spectrum of a peptide correlates an amide proton with its directly bonded amide nitrogen, resulting in cross-peaks for each amide

group in the protein. The chemical shifts of these resonances are sensitive to changes in the local chemical environment caused by the structure of the protein, thus yielding a fingerprint for the folded form of the protein.(40) Changes in protein structure will manifest as changes in the chemical shifts of the cross peaks in the spectrum, providing a convenient and sensitive method for interrogating the protein conformation.

The ^{15}N-HSQC spectra of Gb1 supernatants before and after interaction with kaolinite for 24 h at pH 5 and 7 are shown in Figure 1. The unreacted control spectra of the supernatants show the expected resonances corresponding to folded Gb1.(29) The supernatant spectrum of Gb1 incubated with kaolinite at both pHs tested is essentially identical to the corresponding control spectrum (no mineral), indicating that the protein remains folded and adopting its native structure in solution. The intensities of the resonances for Gb1 are reduced in the pH 5 supernatant, indicating that some of the protein is adsorbed to the kaolinite. To determine if the adsorbed protein retained its ability to fold into the native Gb1 structure after interacting with kaolinite, we extracted the protein from the kaolinite, as described in the SI. The resulting Gb1 ^{15}N-HSQC NMR spectrum was identical to that of the control (see SI), indicating that the protein was not chemically modified such that it altered its ability to fold into its native structure after desorption from kaolinite.

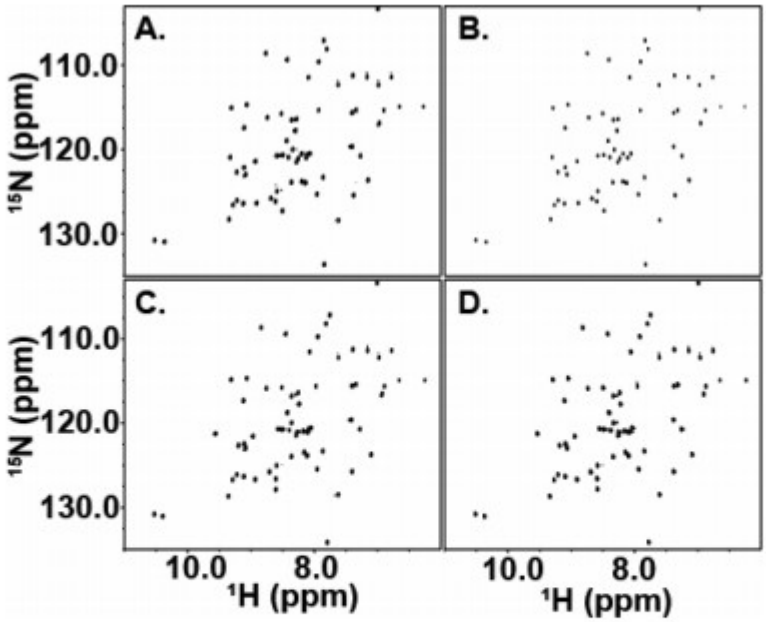


Figure 1. ^{15}N-HSQC spectra of protein kaolinite interactions at pH 5 and pH 7. A. Gb1 without mineral at pH 5. B. Gb1 incubated with kaolinite at pH 5. C. Gb1 without mineral at pH 7. D. Gb1 incubated with kaolinite at pH 7. All kaolinite incubations were conducted for 24 h.

In contrast to kaolinite, analysis of HSQC NMR spectra of the birnessite reaction supernatants revealed that birnessite reacts with the protein to produce soluble peptides. NMR spectra of the birnessite supernatants at pH 5 and pH 7 at various time points are shown in Figures 2 and 3, respectively. At pH 5, the folded protein is rapidly depleted from the sample, shown by the loss of resonances corresponding to the folded protein in solution. Essentially, all of the protein is adsorbed to or reacted with the mineral after 0.5 h under these conditions. The soluble reaction products exhibit poor chemical shift dispersion in the proton dimension, which is consistent with peptide fragments that lack regular secondary and tertiary structure.(40) Addition of 200 μM $MnCl_2$ to 0.4 mg/mL Gb1 caused the expected modest paramagnetic line broadening, but did not induce significant chemical shift perturbations, indicating that soluble Mn(II) alone does not contribute to the reaction (see SI). At pH 7, the protein reacted with the birnessite more slowly, as shown in Figure 3. Most of the protein is still folded and intact after 72 h of interaction with birnessite. Only a small number of weak product resonances are observed after 72 h as shown in Figures 3 and S8. Interestingly, even though only a small number of weak resonances are observed at pH 7, some peaks exhibit similar chemical shifts to those produced at the earliest time points at pH 5. These results indicate that the products produced at pH 7 are similar to those produced early in the reaction at pH 5.

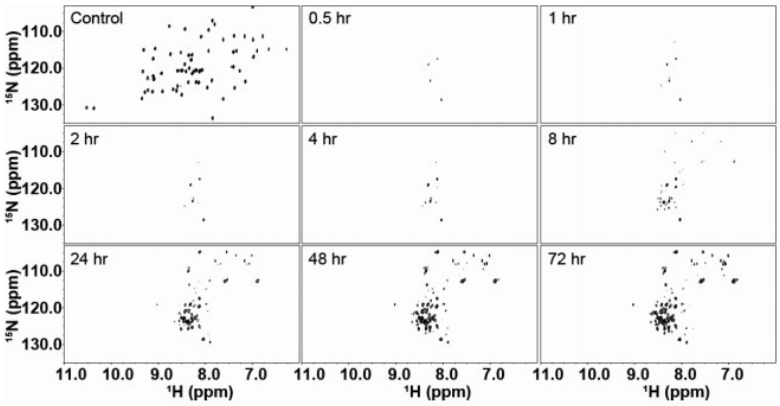


Figure 2. ^{15}N-HSQC NMR spectra of time course of Gb1 degradation in the presence of birnessite at pH 5. Control is 0.4 mg/mL Gb1 protein prepared using the same procedure but omitting the birnessite.

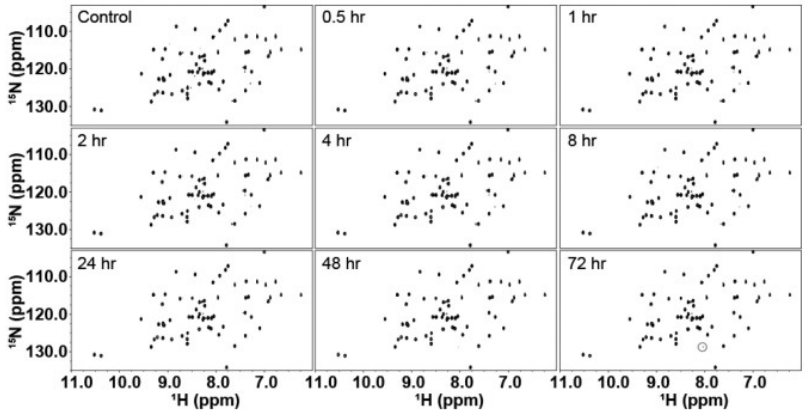


Figure 3. ^{15}N-HSQC NMR spectra of time course of Gb1 degradation in the presence of birnessite at pH 7. Control is 0.4 mg/mL Gb1 protein prepared using the same procedure but omitting the birnessite. At this contour level, one product peak can be observed, indicated by the circle in the 72 h panel.

Our data show that the rate of Gb1 protein adsorption and/or reaction is strongly affected by pH within the range tested. Differences in protein charge likely play an important role in modulating protein adsorption to the birnessite surface. The point of zero charge for birnessite is ~1.3, indicating that the birnessite will have a net negative charge for both pH values tested. Overall, the charge distribution on Gb1 is relatively uniform, with little charge anisotropy, as shown in Figure S10. The theoretical isoelectric point (pI) of Gb1 is ~4.5, indicating that the protein is less negatively charged at pH 5 (charge ~−2.0) when compared to pH 7 (charge ~−4.1). Increased protein adsorption at pH 5 could be the result of reduced electrostatic repulsion caused by reduction of the net negative charge on the protein at lower pH.

To confirm that the loss of resonances corresponding to Gb1 in the ^{15}N-HSQC spectra was caused by protein fragmentation, we also analyzed the samples using SDS-PAGE (see SI) after 24 h of interaction with the minerals. Samples of protein interacted with kaolinite at either pH 5 or pH 7 exhibited the same electrophoretic mobility as the control, indicating that full length Gb1 is present. The bands for the samples reacted with kaolinite at pH 5 were less

intense than those reacted at pH 7, suggesting that more protein was adsorbed to the kaolinite at pH 5 than at pH 7, consistent with the NMR results. Samples interacted with birnessite at pH 5 did not exhibit a band for full length protein, indicating that the reaction supernatant did not contain full length Gb1. A band for full length protein is observed in the pH 7 samples that were interacted for 24 h with birnessite, consistent with the NMR data that indicated the presence of intact protein at pH 7.

The first product peaks to appear at both pH values tested have similar HSQC NMR spectra, suggesting that the product(s) released from the surface during the reaction of birnessite with protein contain the same amino acid sequences. To determine the identity of the initial peptide product that gives rise to the strongest resonances in the HSQC spectrum of the birnessite pH 5 supernatants at the early time points (0.5 h), we analyzed the products using 3D NMR techniques. We assigned the four strongest backbone amide resonances observed in the 0.5 h sample to the sequence TVTE using an HNCACB experiment (see SI). These amino acid residues correspond to the C-terminal four residues of Gb1. The N-terminal residue of a peptide or protein is often not observed in HSQC NMR due to increased chemical exchange with the solvent. Therefore, we used an HNCO triple resonance experiment, which correlates the amide proton with the carbonyl carbon of the preceding residue, to determine if an additional residue was present on this peptide that would correspond to the N-terminal residue of the peptide. We observed a resonance in the HNCO spectrum corresponding to the Threonine at position 53 (T53), indicating that the peptide bond between Phenylalanine 52 (F52) and T53 is intact. Thus, the first peptide products released from the surface during Gb1 degradation by birnessite are derived from the C-terminus of the protein and include the 5-mer peptide FTVTE. We were also able to identify two additional peptides from the 3D NMR data, the 4-mer LKGE and the 5-mer EAVDA, which appeared at later time points. These regions of the protein are highlighted in Figure 4. Finally, aromatic amino acids are often particularly susceptible to oxidation. The ^{15}N-HSQC resonance corresponding to the tryptophan indole ring usually appears shifted well downfield, typically around 10.5 ppm in ^{1}H and 130 ppm in ^{15}N, in both folded and unfolded states. In our data, the control spectra show evidence for the presence of the tryptophan indole ring in Gb1. However, the resonance corresponding to the tryptophan indole ring is not observed in any of the birnessite reactions at pH 5, indicating that either the indole ring has been chemically modified or that the tryptophan containing peptides are still bound to the mineral surface under these reaction conditions.

Oxygen (O_2) plays a key role in some mechanisms of protein degradation. (41) Therefore, we determined whether the extent to which birnessite mediated degradation of Gb1 takes place is different in the absence of oxygen. A comparison of supernatants from reactions of the protein with birnessite carried out with and without oxygen is shown in Figure 5. We found that the spectra of the reaction products at 1 and 24 h with oxygen

are essentially identical to those observed without oxygen. This shows that the reaction does not depend on the presence of oxygen. Thus, fragmentation mechanisms that depend on oxygen availability, such as peroxyl radical based mechanisms,(42) are unlikely to play a significant role in the mechanism of Gb1 protein degradation by birnessite.

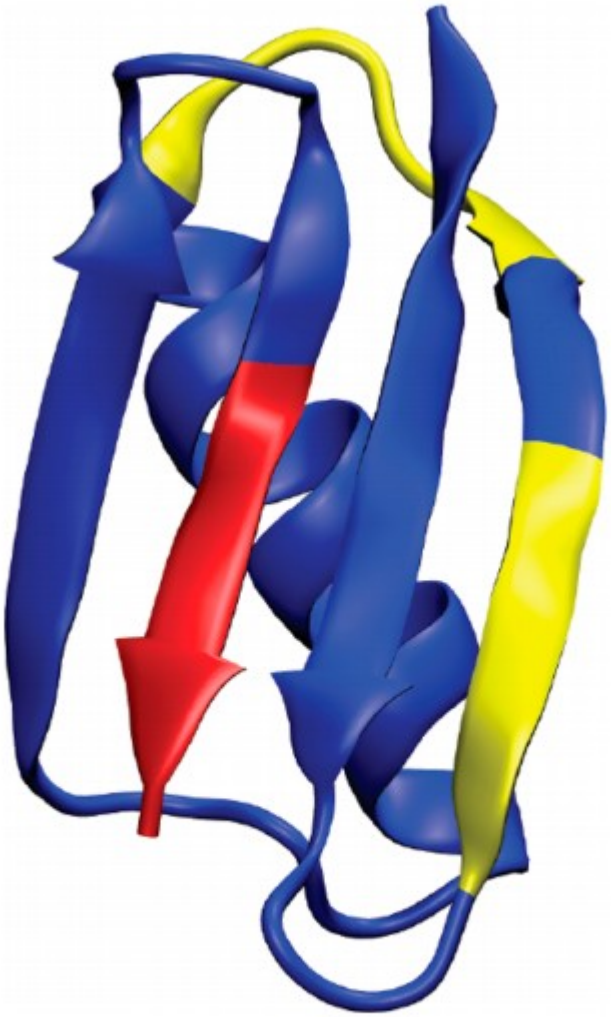


Figure 4. Structure of Gb1 protein showing the initial peptide released during the reaction in red. The NMR identified peptides that are released later in the reaction are shown in yellow. The rest of the protein is shown in blue. Structural coordinates obtained from PDB record 1GB1.

Previous studies have shown that reactions of small organic compounds with birnessite result in reduction of Mn(III,IV) to soluble Mn(II).(16) If the mechanism of protein reaction with birnessite were similar to the reaction with small organic compounds, where birnessite acts as an oxidant, then production of Mn(II) would be expected. We used EPR spectroscopy to determine if Mn(II) was produced by the reaction of protein with birnessite.

EPR analysis of the birnessite reaction supernatants is shown in Figure 6. The data show that the concentration of Mn(II) in the pH 5 solution remains below the detection limits (less than ~100 nM) during 72 h of reaction with birnessite. No significant differences in the concentrations of Mn(II) were observed between birnessite reactions with protein and without protein. We then performed a series of experiments to ensure that the supernatant did not contain manganese in an EPR silent form. To determine if manganese was present as Mn(III) or Mn(IV), we supplemented the supernatant with 1 mM ascorbic acid, which will reduce Mn(III) and Mn(IV) to Mn(II).(17) The addition of ascorbic acid had little impact on the EPR spectrum, indicating that there was little to no Mn(III) or Mn(IV) in the supernatant. To ensure that the soluble reaction products did not interfere with Mn(II) detection by EPR, we spiked a portion of the reaction supernatant with 1 μM Mn(II). The Mn(II) signal was evident and unchanged over a 24 h period, indicating that the supernatant contained no species that would prevent Mn(II) detection by EPR. The addition of 0.4 mg/mL Gb1 to 200 μM $MnCl_2$ also had no impact on the Mn(II) signal, indicating that intact Gb1 does not interfere with Mn(II) detection by EPR spectroscopy (see SI). Analysis of samples prepared under anaerobic conditions show that oxygen does not impact the amount of Mn(II) observed during the reaction of Gb1 with birnessite, indicating that Mn(II) is not reoxidized by oxygen during these reactions. These data imply that either little manganese was solubilized or that any released manganese was readsorbed(43, 44) by the birnessite.

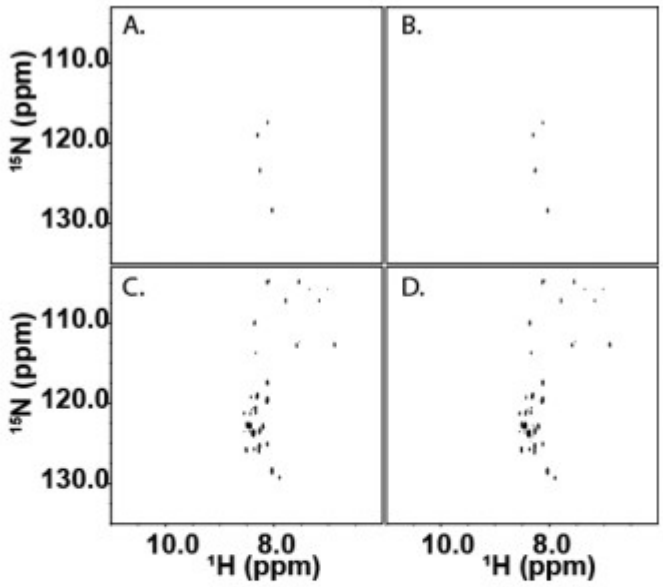


Figure 5. Comparison of birnessite mediated protein degradation with and without oxygen at pH 5. A. Sample collected after 1 h of interaction with birnessite under aerobic conditions. B. Sample collected after 1 h of interaction with birnessite under anaerobic conditions. C. Sample collected after 24 h of interaction with birnessite under aerobic condtions. D. Sample collected after 24 h of interaction with birnessite anaerobic conditions.

Taken together, our observations suggest that the mechanism of protein degradation by birnessite is fundamentally different from that observed for small organic compounds. When birnessite reacts with many small organic compounds, it is a reactant, oxidizing organic compounds and producing Mn(II).(16) In the case of Gb1 protein, Mn(II) is not produced in significant quantities, suggesting that the birnessite is not acting as an oxidant but is acting catalytically. Manganese dioxide, and several other metal oxides, have been shown to catalyze the hydrolysis of *p*-nitrophenyl phosphate,(45, 46) establishing the fundamental ability of metal oxides, and birnessite in particular, to catalyze hydrolysis reactions. The reaction conditions that promote hydrolysis of organophosphates by birnessite and other metal oxides, such as temperature and pH, are similar to the reaction conditions employed in our study. The observation that birnessite catalyzes the hydrolysis of organophosphates under conditions similar to those where we observe protein degradation supports the hypothesis that birnessite catalyzes the hydrolysis of peptide bonds. Although it is possible that the birnessite could be adsorbing Mn(II) produced during the reaction with protein, previous studies have shown that Mn(II) adsorption by birnessite is modest at acidic pH's, similar to the reaction conditions where we observe birnessite mediated protein fragmentation in our study.(44) In addition, previous reports have shown that Mn(II) can react with birnessite and alter

the XRD pattern of the birnessite.(43, 47, 48) In our case, we found that the XRD patterns of the birnessite before and after reaction with Gb1 are essentially identical (see SI), further indicating that little Mn(II) is produced during the reaction. Therefore, we propose that degradation of Gb1 by birnessite is the result of hydrolysis of the peptide bond catalyzed by the birnessite surface. A detailed investigation into the mechanistic and catalytic role of birnessite during Gb1 protein degradation requires further study, and is currently underway.

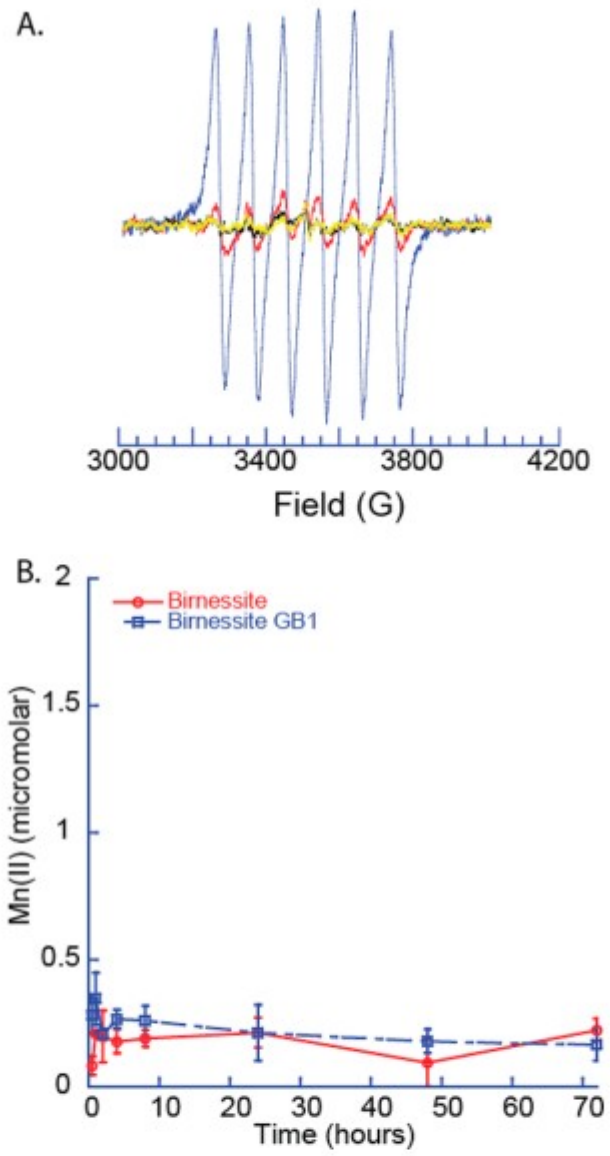


Figure 6. EPR analysis of Mn(II) production. A. EPR spectra supernatant from the pH 5 birnessite reaction with Gb1 at 24 h under various conditions. Black: Reaction supernatant. Red: Reaction supernatant spiked with 1 μM $MnCl_2$. Yellow: Reaction supernatant with 1 mM ascorbate added. Blue: 10 μM $MnCl_2$ concentration standard. B. Mn(II) concentrations measured by EPR spectroscopy over time. Each time point is the average from three sample replicates, error bars are one standard deviation. Samples were prepared either with Gb1 protein (□) or without Gb1 protein (○) at pH 5.

Previous studies involving phyllosilicates and other solid supports have found that some proteins retain enzymatic activity when bound to these surfaces and that this binding may protect these biomolecules from degradation.(49,

50) Our data shows that interaction with kaolinite does not result in degradation of the Gb1 protein under the conditions tested. We also observed that protein extracted from the surface of the kaolinite retained its ability to fold into the native state, indicating that the protein is not chemically modified by binding to kaolinite in a way that would prevent the protein from maintaining its structure dependent functionality in solution when released. This observation does not rule out the possibility of reversible conformational changes occurring at the surface. In contrast, our data show that the oxide mineral birnessite, which is also found in the clay fraction of soils, is not protective and instead can induce degradation and fragmentation of Gb1 protein. A previous report has shown that prion protein and other cellular proteins are degraded in the presence of birnessite,(19) consistent with the data presented here. Our data also show that peptide fragments that are produced during birnessite mediated degradation of Gb1 are released into solution where they could be taken up and either incorporated or further degraded by soil microbes or plants. Importantly, we observe Gb1 protein degradation by birnessite at room temperature and pH values similar to those found in natural soils, suggesting that the reaction is fundamentally possible in the environment. Finally, we find that birnessite is not reduced to Mn(II) in reactions with the protein, apparently acting as a catalyst of hydrolysis and not as a reactant in an oxidation reaction, as is the case with other organic molecules. Thus, our results indicate that the impact of minerals on proteins is dependent on the types of minerals present.

Our findings highlight the potential influence abiotic protein degradation could have on soil nitrogen turnover and bioavailability. In most terrestrial ecosystems, the bioavailability of nitrogen is one of the primary factors limiting growth with the vast majority of the total soil nitrogen present as proteinaceous material.(51) Microorganisms and plants can use organic N compounds, including small peptides, as N sources.(52-54) It has been posited that the formation of dissolved organic N rather than ammonia production can control N dynamics in soil.(55) In many ecosystems, the process controlling N-supply is the depolymerization of N-containing compounds due to the activity of enzymes, such as extracellular proteases released by microorganisms.(54) Our data show how mineral-mediated, abiotic processes can produce small peptides similar in size to those that can be utilized by plants and microorganisms(56) and contribute to the availability of organic N to plants and microorganisms. These processes could be particularly important in many humid forest soils with oxic/anoxic transition zones where biogenic manganese oxides are produced by some microorganisms.(57) The presence of reactive metal-oxides such as birnessite could either increase bioavailability by fragmenting proteinaceous material, or conversely, reduce bioavailability by contributing to the fragmentation and deactivation of extracellular proteases. Thus, we propose a complex role for the mineral matrix as a control of nitrogen availability in the environment: generation of a supply of protein fragments for direct

uptake by the soil biota, and control of protease functionality through either adsorption or fragmentation of proteases and/or their protein substrates.

Acknowledgments

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Abbreviations

NMR	Nuclear Magnetic Resonance
EPR	Electron Paramagnetic Resonance
HSQC	Heteronuclear Single Quantum Correlation
DSS	4,4-Dimethyl-4-silapentane-1-sulfonic Acid
Gb1	B1 domain of protein G from group G streptococcus

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